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Phylogenetic Relationships between Solitary and Colonial Ascidians, as Inferred from the Sequence of the Central Region of their Respective 18S rDNAs

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Abstract. Ascidians (tunicates) are primitive chordates. In spite of their elevated phylogenetic position in the animal kingdom, ascidians have evolved a varied reproductive repertoire; some of them live as individuals (solitary ascidians), while others form colonies (colonial ascidians). Colonial ascidians propagate asexually by budding and strobilation, and they have an extensive capacity for regeneration. However, the orthodox taxonomic classification of ascidians categorizes them into two major groups (the orders Enterogona and Pleurogona), irrespective of their solitary or colonial life style. To examine whether the orthodox classification of ascidians is substantiated by molecular phylogeny, the complete nucleotide sequence of a region of about 1000 base pairs in the central part of their respective 18S rDNAs was determined, and the sequences were compared among five solitary and three colonial ascidians. The phylogenetic tree deduced from these results suggests that the three species of Enterogona and the five species of Pleurogona examined form discrete and separate groups irrespective of their potential to form colonies. Therefore, a solitary or colonial life style is likely to have developed independently after the divergence of the two major groups of ascidians.

Introduction

Ascidians (subphylum Urochordata, class Ascidiacea), or sea squirts, are ubiquitous, sessile marine animals that can be classified as members of about 2,300 species. Since a tadpole-type larva with a well-organized notochord is formed, ascidians are regarded as one of the most primitive types of chordate (Kowalevsky, 1986; Berrill, 1955). Recent studies of the molecular phylogeny of the animal kingdom by comparison of sequences of 5S rRNA (Hori and Osawa, 1987) and 18S rRNA (Field *et al.*, 1988) support this view; namely, ascidians are more closely related to vertebrates than to invertebrates. This view is also supported by recent studies of the structure of genes for muscle actin (Kusakabe *et al.*, 1992). In spite of such an elevated phylogenetic position in the animal kingdom, ascidians have evolved a wide variety of patterns and modes of development. Some of them live as individuals (solitary ascidians), while others form colonies (colonial ascidians). Solitary ascidians propagate exclusively by sexual reproduction, whereas colonial ascidians reproduce both sexually, and asexually by budding and strobilation (Berrill, 1935; Nakauchi, 1982). In addition, colonial ascidians have an extensive capacity for regeneration. On the basis of these differences, ascidians were initially classified as solitary (or simple) or as members of colonial (or compound) groups (Savigny, 1816). This classification is rather convenient for explanations of various phenomena in the fields of developmental and cell biology.

The modern and more orthodox taxonomic classification of ascidians is based mainly on the morphology of the branchial sac and the gonad, and at present, ascidians are subdivided into two orders, the Enterogona and Pleurogona (Lahille, 1886; Huus, 1940; Berrill, 1936, 1950; Millar, 1966; Kott, 1969; Monniot and Monniot, 1973). The Enterogona, in which each individual has a single gonad, contains two subgroups, namely, Aplousobran-

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Table 1

Distribution of PCR, cloning, and sequencing errors

Position in sequence	Individual 1		Individual 2	
	Clone 1	Clone 2	Clone 1	Clone 2
297	C	C	G	C
328	A	A	C	A
352	G	C	G	G
511	G	A	G	G
525	T	T	T	C
595	C	T	T	T
773	T	T	T	C
802	G	A	A	A
857	G	G	A	G

chiata and Phlebobranchiata; members of the former subgroup have a simple branchial sac, and members of the latter have a branchial sac with longitudinal vessels. The Pleurogona, in which each adult has a pair of gonads, contains two subgroups, namely, the Stolidobranchiata and Aspiraculata; members of the former subgroup have a folded branchial sac with longitudinal vessels, and members of the latter live in the deep sea and are macrophagous. Some aspects of the taxonomical interpretation of Aspiculata remain controversial (Kott, 1969; Monniot and Monniot, 1973).

Nucleotide sequence data provide new insight into metazoan phylogenetic relationships and complement the extensive data obtained by paleontological, morphological and developmental analyses. In particular, the sequences of 16–18S and 23–28S ribosomal RNA or DNA provide molecular markers that are useful in attempts to evaluate both long- and short-range phylogenetic relationships within the animal kingdom (Field *et al.*, 1988; Lake, 1990; Christen *et al.*, 1991). In the present investigation, we determined the sequence of a region of about 1000 base pairs (bp) in the central part of the 18S rDNA from eight ascidians, including three colonial species. By comparing these sequences, we analysed the phylogenetic relationship among these ascidians. Our main interest was in determining whether the modern, orthodox taxonomic classification is supported by the sequence data from 18S rDNA, and whether the life style of solitary and colonial ascidians developed independently of the divergence of the two major groups of ascidians.

Materials and Methods

Ascidians

Eight species of ascidians, three from the order Enterogona and five from the order Pleurogona, were examined in this study. The former included *Ciona savignyi, Perophora japonica, Ascidia sydneiensis samea,* and the latter included *Halocynthia roretzi, Pyura mirabilis, Styela clava, Polyandrocarpa misakiensis,* and *Symplegma reptans. Per. japonica, Poly. misakiensis* and *Sym. reptans* are colonial ascidians; the five others are solitary ascidians.

```
        143
18S rRNA:GTTTACTTTGAAAAAATTAGAGTGTTCAAA-CAGGCTGTTCGCCTGCATAGTGTTGCATGGAATAATGGAAT
18S rDNA:.............................G.....................................

AGGACCTCGGTTCTATTTTGTTGGTTTTCGGAGCACGAGGTAATGATTAAGAGGGAC?GACGG?GCCGTCCG
.....................................................A.....G.G......

T?CTCTGCCGTTAGAGGTGAAATTCTTGGATCGGCGGAAGACGAACTACTGCGAAAGCATTCGCC?AGAATG
.A.....................................................A......

TTTTCTTT?ATC?AGA-CGAAAGTCAGAGGTTCGAAGACGATCAGATACCGTCCTAGTTCTGACTATAAACG
........A...A...G.....................................................

                                                                    504
ATGCCAACTAGCGATCGGGAGGCGTTACCATGACGACCTTTCCG?CAGCTTCCGGGAAACCAAAGTCTTTG
.....................................G.....................
```

Figure 1. Comparison between the nucleotide sequence of a region of the 18S rDNA of *Styela clava*, determined in the present study, and that of 18S rRNA, as reported by Field *et al.* (1988). The data for 18S rRNA were kindly provided by Dr. Rudolf A. Raff of Indiana University.

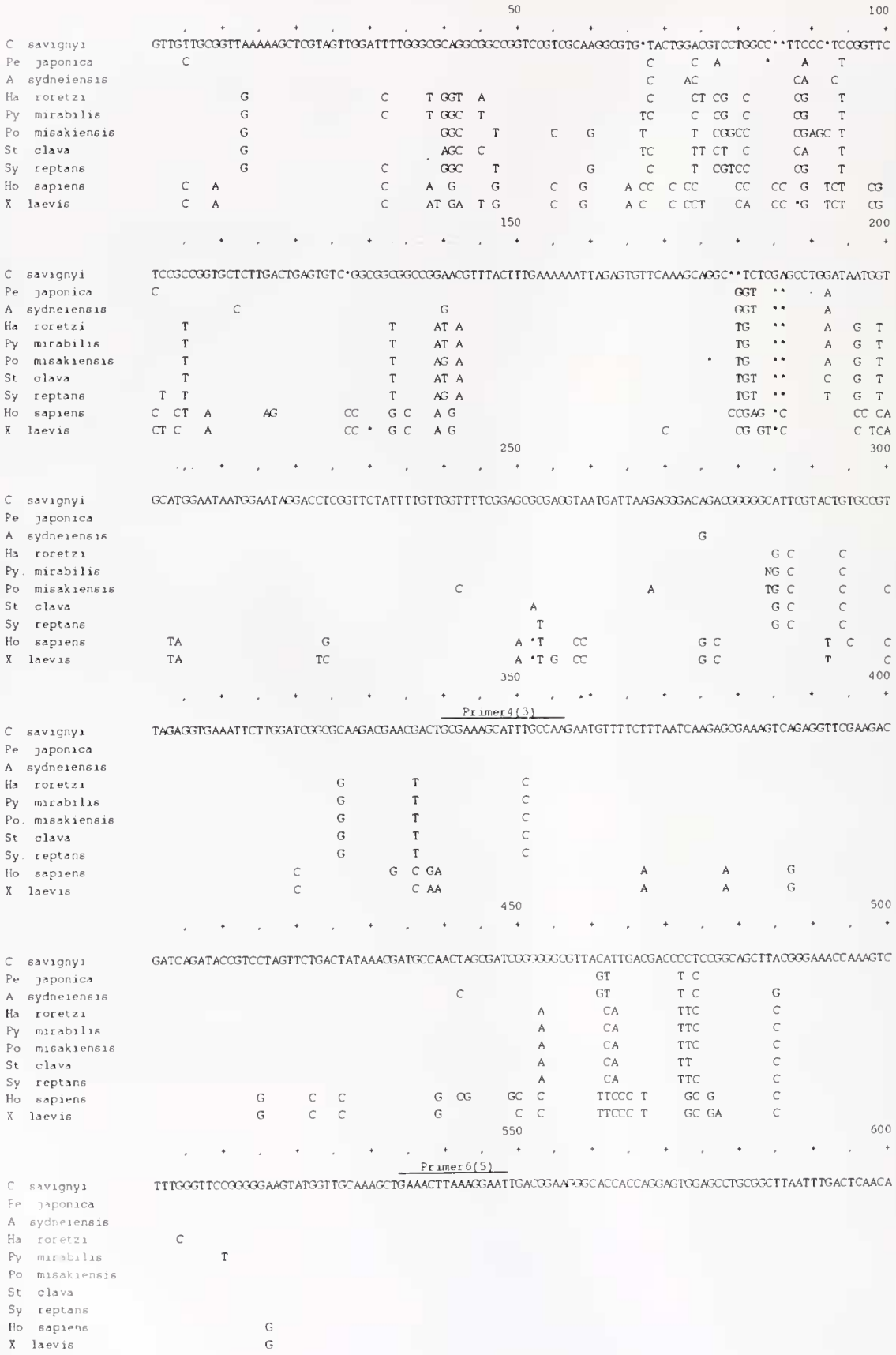
50
100
C savignyi GTTGTTGCGGTTAAAAAGCTCGTAGTTGGATTTTGGGCGCAGGCGGCCGGTCCGTCGCAAGGCGTG*TACTGGACGTCCTGGCC**TTCCC*TCCGGTTC
Pe japonica
A sydneiensis
Ha roretzi
Py mirabilis
Po misakiensis
St clava
Sy reptans
Ho sapiens
X laevis
150
200
C savignyi TCCGCCGGTGCTCTTGACTGAGTGTC*GGCGGCGGCCGGAACGTTTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGC**TCTCGAGCCTGGATAATGGT
Pe japonica
A sydneiensis
Ha roretzi
Py mirabilis
Po misakiensis
St clava
Sy reptans
Ho sapiens
X laevis
250
300
C savignyi GCATGGAATAATGGAATAGGACCTCGGTTCTATTTTGTTGGTTTTCGGAGCGCGAGGTAATGATTAAGAGGGACAGACGGGGGCATTCGTACTGTGCCGT
Pe japonica
A sydneiensis
Ha roretzi
Py. mirabilis
Po misakiensis
St clava
Sy reptans
Ho sapiens
X laevis
350
400
Primer4(3)
C savignyi TAGAGGTGAAATTCTTGGATCGGCGCAAGACGAACGACTGCGAAAGCATTTGCCAAGAATGTTTTCTTTAATCAAGAGCGAAAGTCAGAGGTTCGAAGAC
Pe japonica
A sydneiensis
Ha roretzi
Py mirabilis
Po. misakiensis
St clava
Sy. reptans
Ho sapiens
X laevis
450
500
C savignyi GATCAGATACCGTCCTAGTTCTGACTATAAACGATGCCAACTAGCGATCGGGGGGCGTTACATTGACGACCCCTCCGGCAGCTTACGGGAAACCAAAGTC
Pe japonica
A sydneiensis
Ha roretzi
Py mirabilis
Po misakiensis
St clava
Sy reptans
Ho sapiens
X laevis
550
600
Primer6(5)
C savignyi TTTGGGTTCCCGGGGAAGTATGGTTGCAAAGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACA
Pe japonica
A sydneiensis
Ha roretzi
Py mirabilis
Po misakiensis
St clava
Sy reptans
Ho sapiens
X laevis

Figure 2.

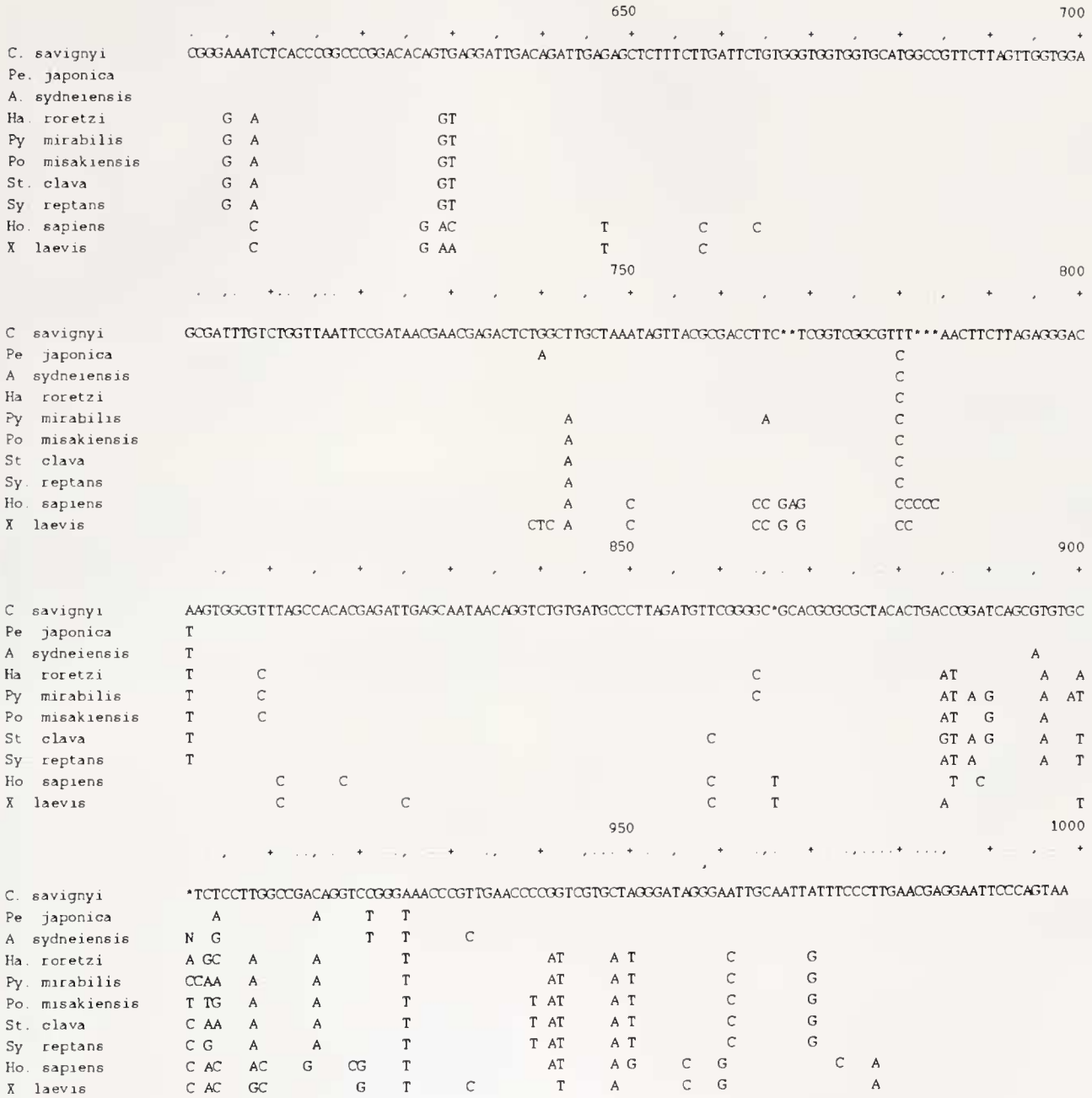

Figure 2. Alignment of the central regions of the 18S rDNAs from eight ascidians analysed by the present study, *Homo sapiens* and *Xenopus laevis* (Neefs *et al.*, 1990). All the bases are shown for *Ciona savignyi* and only bases different from these are shown for other species. Asterisks indicate deletions. The four primers used for sequencing are shown by underlining.

Per. japonica, *Poly. misakiensis*, and *Sym. reptans* were generously provided by Dr. K. Kawamura, Kochi University, Japan.

Isolation of DNA

The gonad or the whole animal was frozen in liquid nitrogen and kept at −80°C until used. Frozen and powdered samples were lysed in TE buffer (10 m*M* Tris-HCl, 0.1 *M* EDTA, pH 8.0) that contained 0.5% sodium dodecyl sulfate. After digestion of samples with proteinase K (100 μg/ml) at 50°C for 3 h, DNA was extracted with phenol and precipitated in ethanol and an equal volume of 5.0 *M* ammonium acetate. Samples resuspended in TE buffer were further purified by treatment with RNase A

Table II

Structural similarity and evolutionary distance data for ascidian 18S rDNA sequences

Species	*C.s.*	*P.j.*	*A.s.s.*	*H.r.*	*Py.m.*	*Po.m.*	*St.c.*	*Sy.r.*	*H.s.*	*X.l.*
Ciona savignyi		0.0218	0.0250	0.0641	0.0697	0.0697	0.0641	0.0675	0.1258	0.1233
Perophora japonica	21		0.0166	0.586	0.0630	0.0675	0.0586	0.0630	0.1221	0.1221
Ascidia sydneiensis samea	24	16		0.0653	0.0709	0.0720	0.0664	0.0686	0.1233	0.1245
Halocynthia roretzi	60	55	61		0.0145	0.0271	0.0239	0.0229	0.1331	0.1355
Pyura mirabilis	65	59	66	14		0.0271	0.0208	0.0229	0.1355	0.1380
Polyandrocarpa misakiensis	65	63	67	26	26		0.0282	0.0197	0.1367	0.1417
Styela clava	60	55	62	23	20	27		0.0208	0.1355	0.1392
Symplegma reptans	63	59	64	22	22	19	20		0.1318	0.1355
Homo sapiens	113	110	111	119	121	122	121	118		0.0454
Xenopus laevis	111	110	112	121	123	126	124	121	43	

The lower-left half of the table gives the number of substitutions in which gaps are not included. The upper-right half of the table gives the evolutionary distance values (average number of nucleotide substitutions per sequence position) determined by the Jukes and Cantor (1969) formula.

(20 μg/ml) at 37°C for 1 h and then precipitated in ethanol.

Amplification of the central region of 18S rDNA

A region of about 1000 bp from the central part of 18S rDNA was amplified by the polymerase chain reaction (PCR; Saiki *et al.*, 1988) in a Perkin Elmer Cetus thermal cycler. Amplifications were performed in 100 μl of 50 m*M* KCl, Tris-HCl (10 m*M*, pH 9.0), 0.1% Triton X-100, with 0.2 m*M* each dNTP, 100 p*M* primer, template DNA (10–100 μg) and 2 U *Taq* DNA polymerase (Promega). Primer-1 (5′-CAG(CA)CCCGCGGT-AAT(TA)C-3′) and Primer-2 (5′-ACGGGCGGTGTGT(AG)C-3′), the latter being identical to Primer C of Field *et al.* (1988), were used for amplification. The temperature regimen for

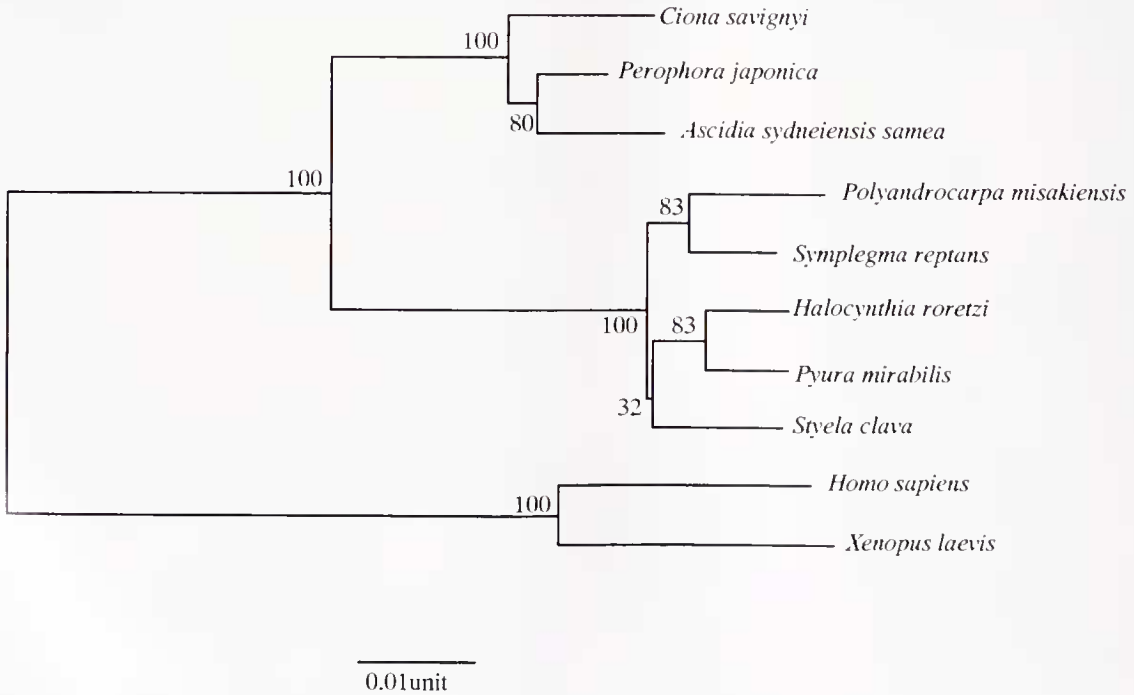

Figure 3. Phylogenetic tree of eight ascidians, as deduced by the neighbor-joining method. The scale bar indicates an evolutionary distance of 0.01 nucleotide substitution per sequence position. Numbers at each branch indicate the percentage of times a node was supported in 200 bootstrap pseudoreplications by neighbor joining method.

30 cycles was 1 min at 92°C, 2 min at 55°C, and 3 min at 72°C.

Cloning and sequencing of the amplified DNA

Amplified DNA was purified by electrophoresis in 0.8% agarose, inserted into the *Bam*HI site of the vector pBluescriptII SK(+) (Stratagene), and cloned. Sequencing was performed by the dideoxy chain-termination method (Sanger *et al.*, 1977) using Sequenase ver 2.0 (USB) and [^{35}S]-dATP (Amersham). In addition to Primers 1 and 2, Primer-3 (5′-GCGAAAGCATTTGCCAA-3′), Primer-4 (antisense analog of Primer-3), Primer-5 (5′-GAAAC-T(TC)AAAGGAAT-3′), and Primer-6 (antisense analog of Primer-5) were used for sequencing.

Comparison of sequences and inferences about phylogeny

Sequences were aligned on the basis of maximum nucleotide similarity. Using the aligned sequences, evolutionary distance values were calculated pair-wise as described by Jukes and Cantor (1969). The phylogenetic tree was inferred from an analysis of results by the neighbor-joining method of Saitou and Nei (1987). The degree of support for internal branches of the tree was further assessed by bootstrapping (Felsenstein, 1985).

Results and Discussion

Validity of the methodology

Genomic DNA was extracted from each of eight ascidians. The central region of 18S rDNA was amplified by PCR using synthetic oligonucleotide primers (Primers 1 and 2). Amplification yielded a single band of DNA of about 1000 bases in length. The product of PCR was subsequently inserted into the *Bam*HI site of pBluescriptII SK(+) and cloned. The complete nucleotide sequence of the fragment was determined by the dideoxy termination-chain method using six primers. The sequences we determined in the present study correspond to positions 656–1643 in the sequence of human 18S rRNA (Neefs *et al.*, 1990).

Amplification of certain DNA fragments by PCR with the aid of Taq DNA polymerase may result in a few mismatched bases per 1 kilobase of DNA. Such misamplification causes confusion in the interpretation of results. To examine this possibility, we first selected two individuals of *H. roretzi*, and determined the nucleotide sequences of the 1000-bp-long portion of 18S rDNA twice for each specimen by separate amplification and cloning. As shown in Table I, there were four mismatches in the nucleotide sequences when two independent analyses of individual 1 were compared, and five mismatches in the case of individual 2. However, no mismatches occurred at identical positions in the two individuals. Therefore, one can distinguish changes in nucleotide sequence caused by misamplification during the PCR from those that are specific to individuals in the natural population. In this study we examined at least two clones of each of eight species. When the nucleotide sequences of the two clones were identical, we took this sequence as that of this species. When the sequences were not identical, we performed a third cloning.

The partial nucleotide sequence of 18S rRNA of the ascidian *Styela clava* has already been reported (Field *et al.*, 1988), and the relevant data were kindly provided by Dr. Rudolf A. Raff of Indiana University. To examine still further the validity of the present method, we determined the nucleotide sequence of the 18S rDNA of this species and compared it with that of the 18S rRNA. The nucleotide sequences of two regions of 18S rDNA, each of about 300 bases, were compared with the corresponding sequences in 18S rRNA. The nucleotide sequence of one region of the 18S rDNA was identical to that of a region of the 18S rRNA (data not shown). The result for the other region is shown in Figure 1. Seven bases in the 18S rRNA in this region were not determined in the previous study (Field *et al.*, 1988). We were able to identify all of the bases. We also identified two bases that may have been missed in the previous study. We found that there was a single-base mismatch between the results of the two studies, which may be a difference caused by individuality. Therefore, the present method is apparently valid for an analysis of the molecular phylogeny of ascidians.

Complete nucleotide sequences of rRNA genes including 18S rDNA of the ascidian *Herdmania momus* have been reported by Degnan *et al.* (1990), but we did not include the *H. momus* 18S rDNA sequence in the present study.

Partial nucleotide sequences of the 18S rDNAs from eight ascidians

The complete nucleotide sequences of a region of about 1000 bp in the central part of the 18S rDNA of eight species of ascidians are summarized in Figure 2. As mentioned previously, these sequences correspond to positions 656–1643 in the sequence of human 18S rRNA (cf. Neefs *et al.*, 1990). Alignment of the nucleotide sequences from the eight ascidian species revealed some interesting features. In some regions, the nucleotide sequences are highly conserved; very few changes are evident among the eight species. In particular, the sequences from position 511 to position 742 are completely identical among the five species of the order Pleurogona. In contrast, nucleotide sequences of other regions, such as positions 32–92 and

positions 885–950, are highly variable. Sequences of yet another region vary only moderately. These differences may reflect differences in the functional importance of the regions in the 18S rRNA. In addition, the five species of the order Pleurogona clearly share common nucleotide sequences, as do the three species of the order Enterogona. However, the nucleotide sequences from the two groups are very different, suggesting the early divergence of the two groups of ascidians during evolution.

Phylogenetic relationships

Structural similarity and evolutionary distance values were calculated pair-wise (as described by Jukes and Cantor, 1969) between the sequences aligned in Figure 2 and those of *Homo sapiens* and *Xenopus laevis;* the results are summarized in Table II. A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987), by reference to the distance values of Table II. The phylogenetic tree (Fig. 3) indicates that the eight ascidians examined here can be subdivided into two groups, corresponding to the orders Enterogona and Pleurogona. That is, the relationships between the partial nucleotide sequences of 18S rDNAs support the modern and orthodox classification, but not the classification of ascidians into solitary and colonial groups.

Among species of the order Pleurogona, two pyuride ascidians, namely, *Pyu. mirabilis* and *H. roretzi*, and two styelide ascidians, namely, *Poly. misakiensis* and *Sym. reptans*, form discrete groups, because a high percentage of bootstrap analysis was given. However, the exact position of another styelide, *Styela clava*, was not determined in this study. The Styelidae form one of three families that include both solitary and colonial species (the others are Cionidae and Octacnemidae), and the Styelidae are divided into two subfamilies, namely, the Polyzoinae (colonial) and the Styelinae (solitary). Although the present results suggest the divergence between the two subfamilies, it is uncertain at present whether *Styela clava* is more closely related to the Styelidae or the Pyuridae.

Ascidians were initially classified as solitary and colonial species (Savigny, 1816). Around the turn of the century, however, ascidians were classified, mainly by reference to the morphology of the branchial sac and the gonad, into three major groups: the suborder Aplousobranchiata (colonial ascidians with a simple branchial sac, spicardia present, and gonads located within the gut loop), the suborder Phlebobranchiata (solitary and colonial ascidians with longitudinal vessels within the branchial sac, and gonads located within the gut loop), and the Stolidobranchiata (solitary and colonial ascidians with a folded branchial sac that contains longitudinal vessels, and gonads located to one side of the pharyngeal baskets) (Lahille, 1886; Huus, 1940). Recent investigations into the morphology of internal organs and lifestyles tend to support such a classification (Berrill, 1950; Millar, 1966; Kott, 1969; Monniot and Monniot, 1973). As shown by the present study, the comparison of nucleotide sequences of 18S rDNA also supports this orthodox classification.

In the present study, we included only three of nine families of the order Enterogona and two of five families of the order Pleurogona. The molecular phylogeny, as deduced by comparisons of nucleotide sequences from 18S rDNAs of species within all known families of ascidians is the focus of further investigations.

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